REPORT DOCUMENTATION PAGE

Form Approved OMB NO. 0704-0188

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED (From - To)
19-02-2016	Final Report	1-Oct-2011 - 30-Sep-2015

4. TITLE AND SUBTITLE
Final Report: Living Membranes as Environmental Detectors

5a. CONTRACT NUMBER
W911NF-11-1-0444

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER
611102

6. AUTHORS
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAMES AND ADDRESSES
Tufts University
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS (ES)
U.S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSOR/MONITOR'S ACRONYM(S)
ARO

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
58318-LS.2

12. DISTRIBUTION AVAILIBILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not contrued as an official Department of the Army position, policy or decision, unless so designated by other documentation.

14. ABSTRACT
The work conducted over the course of this program has made significant progress towards the technical objectives outlined in the original proposal. Working with the bacterial cellulose “Living Membrane” system, we have addressed the major design and structural impediments to develop a basic functional system that is specifically responsive to one of the two target pathogens identified in our original proposal. In addition to the core results, we have explored important aspects of the system, including loading capacity, long-term stability, detection capacity, and cloning of the cellulose genetic machinery. We have also identified potential future directions to optimize and

15. SUBJECT TERMS
bacterial cellulose, living membrane, detection, antigen, antagonist, efficacy, devices

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	15. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			David Kaplan
UU	UU	UU	UU		19b. TELEPHONE NUMBER 617-627-3251

Report Title

Final Report: Living Membranes as Environmental Detectors

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The work conducted over the course of this program has made significant progress towards the technical objectives outlined in the original proposal. Working with the bacterial cellulose "Living Membrane" system, we have addressed the major design and structural impediments to develop a basic functional system that is specifically responsive to one of the two target pathogens identified in our original proposal. In addition to the core results, we have explored important aspects of the system, including loading capacity, long-term stability, detection capacity, and cloning of the cellulose genetic machinery. We have also identified potential future directions to optimize and improve on the design of the core functional system developed to date.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received	Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received	Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received	Paper	
09/12/2012	1.00	Guokui Qin, Bruce Panilaitis, Zhongyuan Sun, David Kaplan. A cellulosic responsive "living" membrane, Carbohydrate Polymers (08 2012)

TOTAL: **1**

Number of Manuscripts:

Books

Received Book

TOTAL:

Received Book Chapter

TOTAL:

Patents Submitted

Patents Awarded

Awards

Graduate Students

NAME	PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Names of Post Doctorates

NAME	PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Bruce Panilaitis	0.33	
FTE Equivalent:	**0.33**	
Total Number:	**1**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: .....

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:.....

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Names of Personnel receiving masters degrees

NAME
Total Number:

Names of personnel receiving PHDs

NAME
Total Number:

Names of other research staff

NAME	PERCENT_SUPPORTED
Chuang Du	0.85
FTE Equivalent:	**0.85**
Total Number:	**1**

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

See Attachment.

Technology Transfer

Submissions or publications under ARO sponsorship:
None, manuscript in preparation

Scientific Progress and Accomplishments:

This program has been very productive in terms of validating the stated goals of the proposal as well as establishing the fundamentals to bring the living membrane concept to fruition. Chemical and biological weapons (CBW) present unique challenges with regards to detection and treatment. The rapid onset of medical effects, particularly with chemical agents requires that detection methods are developed that will not only be specific and sensitive, but also provide reliable results very quickly. Current methods generally utilize sophisticated equipment that is fragile, requires significant power supply, or scientific training to operate. In order to successfully address these challenges, the ideal detection method would meet the following criteria:

- Provide specificity to particular chemicals or pathogens with high sensitivity
- Provide rapid and simple to read outputs
- Require little, if any scientific training
- Require little or no power input
- Meet all of the above requirements in a physically robust format able to withstand extremes of temperature, humidity, and other environmental variables

The living membrane systems under development are envisioned for application in a forward operating area by members of the armed forces with limited or no scientific training. The reading of these devices will be a simple binary yes or no as determined by the presence or absence of a fluorescent signal. The use of cellulosic membranes with entrapped recombinant bacteria provides significant advantages over other potential systems:

- Robust nanofibril matrix
- Natural entrapment of target bacteria
- Genetically malleable host organism
- Tailorable cellulose pellicle formulation
- Biologically-driven rather than chemical synthesis

The work conducted in this proposal can be organized into three general areas:

1. Reporter Suite Construction
2. Detection Suite Construction
3. Device Development

Reporter Suite Construction:

As a test system to validate the concept proposed, the reporter protein Green Fluorescent Protein (GFP) was placed under the control of the P_{lac} promoter. In addition the regulatory protein luxR was cloned downstream of the reporter for eventual amplification of the signal. For a subset of experiments, the Red Fluorescent Protein (RFP) was substituted into the reporter operon. To examine the effectiveness of the reporter operon, the experimental protocol described in **Figure 1** was followed. After 4 hours of IPTG induction, GFP or RFP expressions was detectable in a manner dependent on the initial *E. coli* loading (**Figure 2**).

Figure 1: Experimental Protocol for Reporter Operon Analysis. After the cellulose membranes were produced by *G. xylinus* in HS medium for 4 days, *E. coli* cells were inoculated and grown in the same HS medium for three days. The GFP or RFP signal was detected in the cellulose membranes with and without IPTG after 4 hours of induction.

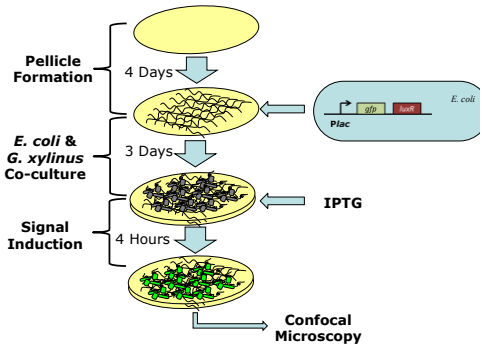
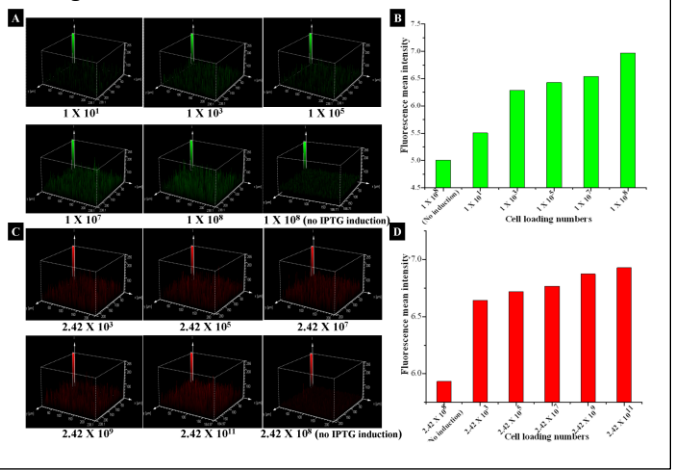


Figure 2: Fluorescence Signal of Reporter Operon. The GFP (A) and RFP (C) fluorescent signals with different loading numbers of E. coli cells were observed by confocal microscopy, compared with the signal expression without IPTG induction. The GFP (B) and RFP (D) fluorescent intensity was further analyzed by Image J software for comparison of fluorescent signal expression with different loading numbers of E. coli cells.



Once the proof of concept was completed, our efforts turned to improving the detection capacity of the constructs by introducing a genetic amplifier loop. To do this, a second plasmid was constructed and introduced into the recombinant *E. coli* which contained a second copy of the reporter protein (GFP or RFP) and an additional copy of the luxR protein. Both of these genes were placed under control of the promoter P_{luxI} which is positively regulated by the luxR protein. When both plasmids are present in the same organism, a single activation of the Plac promoter should induce an amplified signal due to the genetic feedback established (**Figure 3**).

Once the amplifier loop was constructed, it was tested as described above, demonstrating a significant increase in fluorescence signal with the same bacterial loading (**Figure 4**).

Figure 3: Genetic Amplifier Loop. The amplifier consists of a plasmid pGN68 where LuxR has been cloned behind the PluxI promoter. The inducer IPTG will bind to the T7 promoter and control the expression of GFP-LuxR fusion as an output signal. The LuxR produced here was again used to bind to the PluxI promoter, this time as the input signal, and activate secondary expression of GFP-LuxR fusion, which in turn feeds back into the amplifier loop.

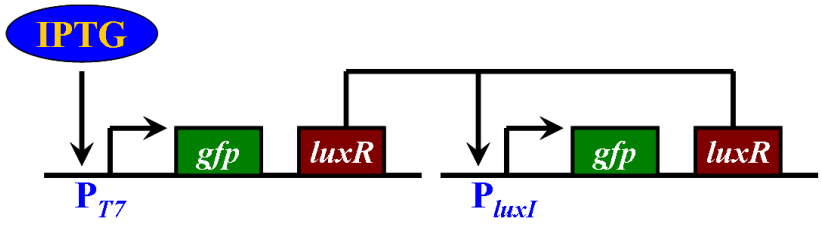
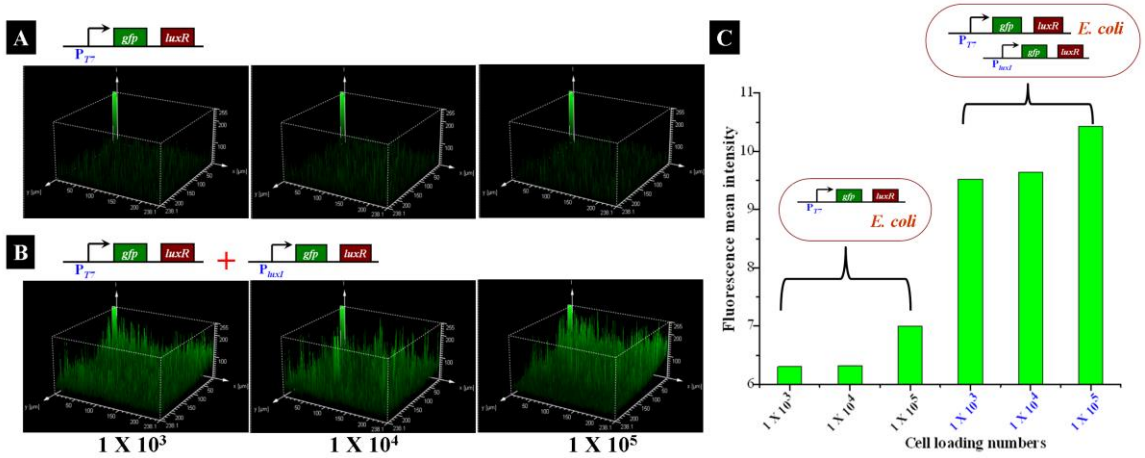


Figure 4: Signal Amplification. Comparison of GFP signal observation in cellulose membrane without (A) and with (B) the positive-feedback genetic amplifier, as well as further comparison analysis of fluorescent intensity by Image J software (C).



Finally, while the results described above were significant, given the ultimate goal of the project is to demonstrate detection via a simple method, it was determined if the signal induced in these studies could be detected by a simple ultraviolet source (**Figure 5**).

While these initial studies validated the basic goals of the study with regards to the reporter suite, the initial construct was driven by the P_{lac} promoter. In order to pair that with the detection suite that will depend on dimerization of the ToxR receptors, the promoter was replaced with P_{CTX} (**Figure 6**).

Figure 5: Signal Amplification. GFP fluorescent signal expressed within *E. coli* BLR (DE3) entrapped in the cellulose membrane observed under UV light.

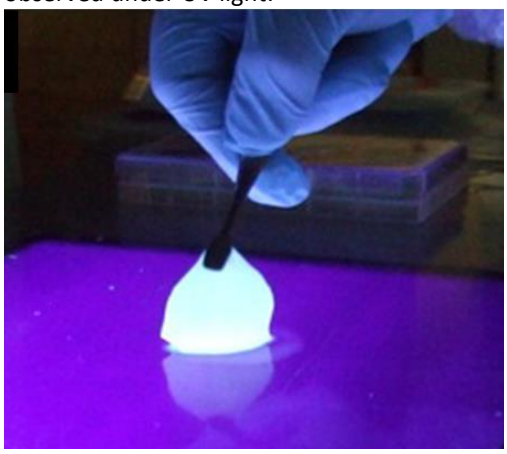
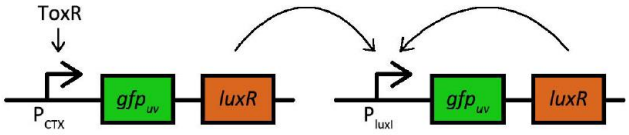


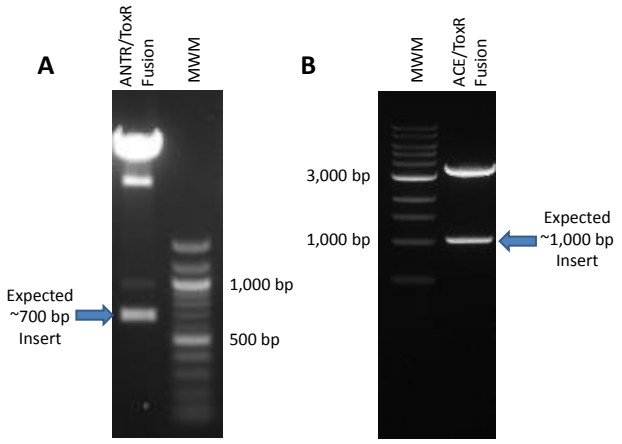
Figure 6: Modified Genetic Amplifier Loop. The genetic amplifier was modified to include the P_{CTX} promoter.



Detector Suite Construction:

The cloning, construction and validation of the pathogen/toxin-specific receptor systems are the crux of this program. For this award, the focus has been on two potential pathogen/toxin systems, the anthrax protective antigen and the class of nerve toxins that target the acetylcholinesterase enzyme. Utilizing a PCR-based strategy, fragments of each gene were cloned and confirmed by sequence. The target region for each gene was determined through a review of the literature identifying the binding site for the desired ligands. These cloned fragments were then combined with a ToxR fragment to generate the full-length fusion receptor. Sequence analysis and restriction digestion (**Figure 7**) was again utilized to confirm the resultant clones. These clones were then transformed into an expression host to validate the presence of fusion protein produced from these constructs. Western blot analysis of each clone

Figure 7: Verification of successful cloning. Restriction digestion of anthrax receptor-ToxR fusion (A) and acetylcholinesterase fusion (B).



the hydrophobic protein isolation sample, indicating its likely association and/or integration with the cell membrane.

While functional assessment of the AChE-ToxR fusion hasn't progressed further at this point, the ATR-ToxR fusion has been transformed into an *E. coli* strain containing the reporter operon we previously developed, under control of the ctx operon. The receptor expression was still dependent on arabinose as an inducer, so cultures were maintained in 1.3 mM arabinose, and then exposed to Anthrax Protectiv e Antigen (PA). Native PA exists as an 83-kDa monomer which is cleaved by the protease furin once bound to the receptor in mammalian systems. This removes some steric hindrance from the PA, and the remaining PA63 allows oligimerization of the anthrax toxin complex (**Figure 9**). For our work we explored the binding capacity and functional outcomes of our fusion receptors with both PA83

indicated the expression of each fusion protein at the appropriate molecular weight (**Figure 8**). In all samples, a protein at approximately 65 kD was detected by the secondary antibody used in these experiments regardless of the induction time of the BL21 cultures. The band at approximately 60 kD, indicated by the red arrow in Figure 8a is at the expected molecular weight of the AChE-ToxR fusion. Similarly, the band visualized at approximately 55 kD is at the appropriate size for the ATR-ToxR fusion receptor. In addition, the receptor is exclusively found in

Figure 8: Expression of ToxR Fusion Receptors. (a) AcHE-ToxR and (b) ATR-ToxR fusion receptor expression in BL21 *E. coli* cultures. The expected band sizes are indicated by the red arrows. In the ATR western (b), the "|" indicates hydrophilic protein isolation while the "O" indicates hydrophobic protein isolation.

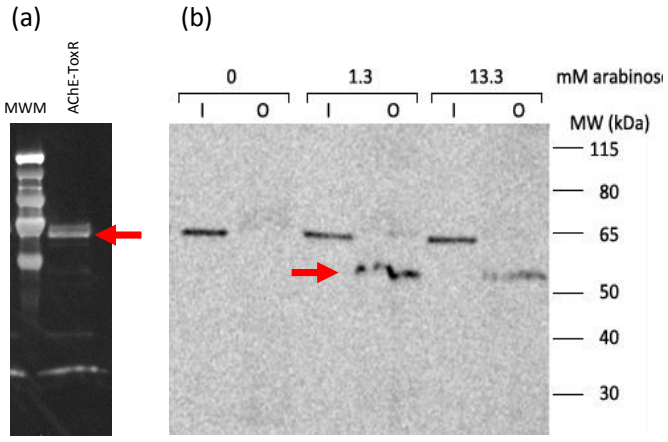
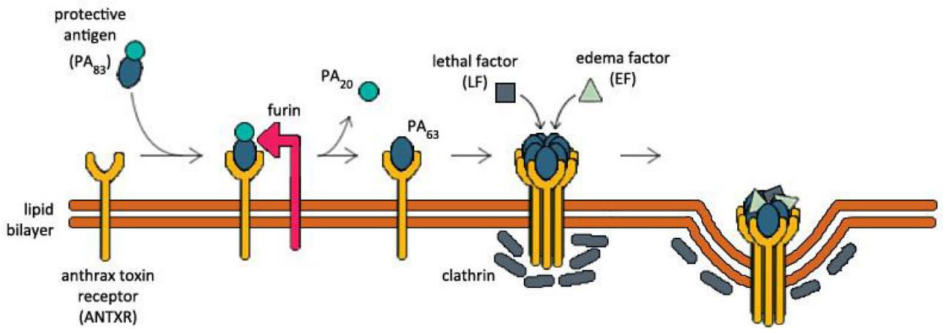


Figure 9: Modified Genetic Amplifier Loop. The genetic amplifier was modified to include the P_{CTX} promoter.



and PA63 (**Figure 10**). Cultures that were not induced with 1.3 mM arabinose are unable to produce the recombinant anthrax receptor. Cells were treated with either no PA or 8 nM of either PA83 or PA63. Following induction with arabinose, cells treated with PA83 exhibited a 19.5% increase in signal compared to the PA-free culture ($p < 0.009$); the sample exposed to PA63 demonstrated a 35.9% increase ($p < 0.003$). While the system clearly will require optimization, the increase in fluorescence in response to specific stimulation by the two protective antigen proteins validates the approach of the work presented here.

Figure 10: Functional Assessment of ATR-ToxR Fusion Receptor. Values reported are the mean ± standard deviation and the error bars represent ± 1 standard deviation. Groups denoted by different letters are statistically different, $p < 0.02$.

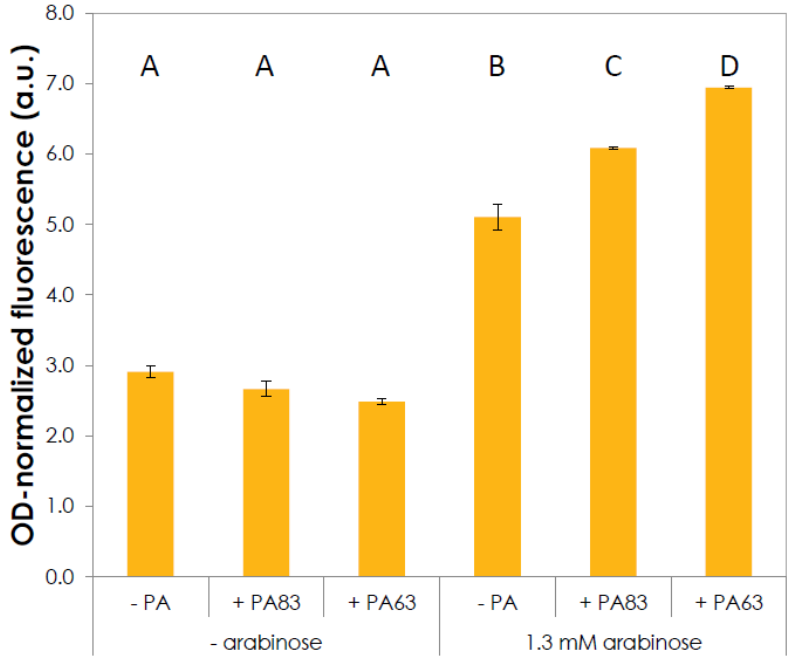
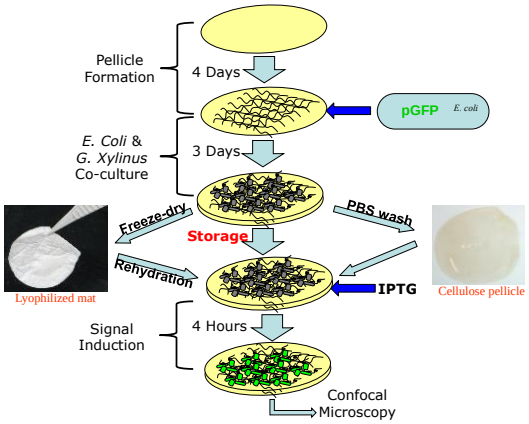


Figure 11: Experimental Protocol for Reporter Operon Analysis. After the cellulose membranes were produced by *G. xylinus* in HS medium for 4 days, *E. coli* cells were inoculated and grown in the same HS medium for three days. The membranes were either lyophilized or kept hydrated for a specified time at various temperatures. Membranes were then rehydrated (for lyophilized samples) or washed with PBS, and GFP or RFP signal was detected in the cellulose membranes with and without IPTG after 4 hours of induction.



Device Development:

For most of our development work, we utilized our construct that has the reporter protein Green Fluorescent Protein (GFP) under the control of the P_{lac} promoter. This construct also has a genetic amplifier loop which contains a second copy of the reporter protein (GFP or RFP) and an additional copy of the luxR protein. Both of these genes were placed under control of the promoter P_{luxI} which is positively regulated by the luxR protein. When activated, a single transcriptional event induces a positive feedback loop with significantly increased fluorescent signal. Using this construct, we have conducted several studies to assess the long term stability of the matrices.

For all stabilization experiments, our basic experimental protocol described in **Figure 11** was followed. Initial studies were conducted for 30 days of storage at room temperature and 4°C. Results indicate that the living membrane is stable over those 30 days without changing the inducibility of the system for GFP or RFP (**Figure 12**). As expected, membranes stored in PBS over that period of time at either temperature

Figure 12: Stability of GFP Living Membrane. Comparison of GFP signal observation in cellulose membranes stored for 30 days at either 4°C or room temperature in wet or lyophilized form.

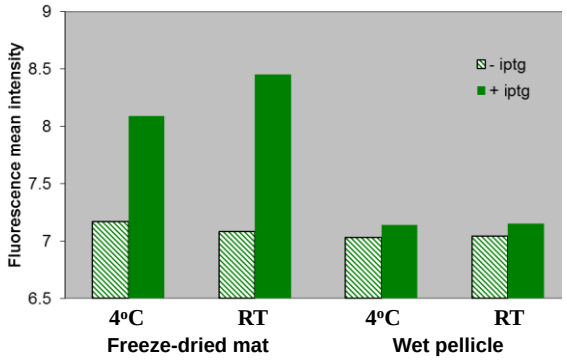
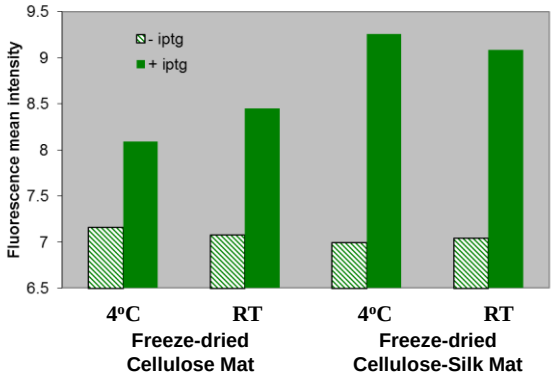


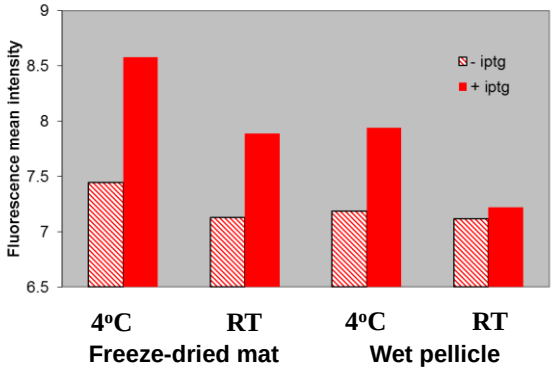
Figure 14: Stability of Silk-Cellulose Composite Living Membrane. Comparison of GFP signal observation in cellulose and cellulose/silk composite membranes stored for 30 days at either 4°C or room temperature in wet or lyophilized form.



resulted in a loss of signal with or without induction. Cellulose matrices that had been lyophilized produced an inducible response to stimulus at either temperature, indicating the capacity to store these samples long term to be used as needed.

This stability of signal was replicated in a construct containing the RFP signal protein as well, although some stability of signal was observed in the wet pellicle stored at 4°C (**Figure 13**).

Figure 13: Stability of RFP Living Membrane. Comparison of RFP signal observation in cellulose membranes stored for 30 days at either 4°C or room temperature in wet or lyophilized form.



In order to develop these matrices as practical point-of-use devices, additional optimization of their long-term stability and the time required for analysis is required. To that end, a living membrane was generated with the addition of silk protein as an additional stabilizing agent. Results after 30 days indicate that the inclusion of silk protein resulted in enhanced stability (**Figure 14**).

In year two, we established the capacity of these membranes to be lyophilized, stored for a period of time at various temperatures, to be reconstituted and respond functionally to control stimuli. However, the rehydration time required was longer than optimal. In order to reduce that time, pellicles were grown with bovine gelatin to facilitate a more rapid rehydration. A range of gelatin was added to the growing pellicle in order to determine the optimal concentration. Based on the gross observation of the pellicles formed (**Table 1**), it was determined that a

Table 1: Gelatin/Cellulose Pellicle Formation

Gelatin Conc. (mg/ml)	Hydrated Observations
0	Pellicle formation
25	Pellicle formation Pellicle similar to controls No freestanding gelatin
50	Pellicle formation Pellicle similar to controls Pure gelatin attached to pellicle
100	No pellicle formation Entire well is solid gelatin

concentration of 25 mg/ml was chosen. Pellicles were grown for 5 days after which the cellulose/gelatin composite was removed from the HS media and washed for approximately 20 seconds in three changes of DI water. These composites were frozen at -20°C overnight and then lyophilized. Samples were rehydrated at 37°C in HS media and weights were taken at 30 minutes, 1 hour, 2 hours, 3 hours and 4 hours. Six samples were prepared at each of these time points for control cellulose samples and the composite samples for a total of 60 samples. Samples were removed from the HS media, drained of surface liquid, and then weighed. These values were compared to the average weights of fully hydrated pellicles (**Figure 15**). The results indicate that the composite pellicles rehydrated more rapidly, and to a more complete hydration status then the control samples after lyophilization.

Figure 15: Membrane Rehydration. The percentage weight is a percentage of the fully hydrated average weight for each of the pellicle samples. N=6/sample, error bars indicate standard error

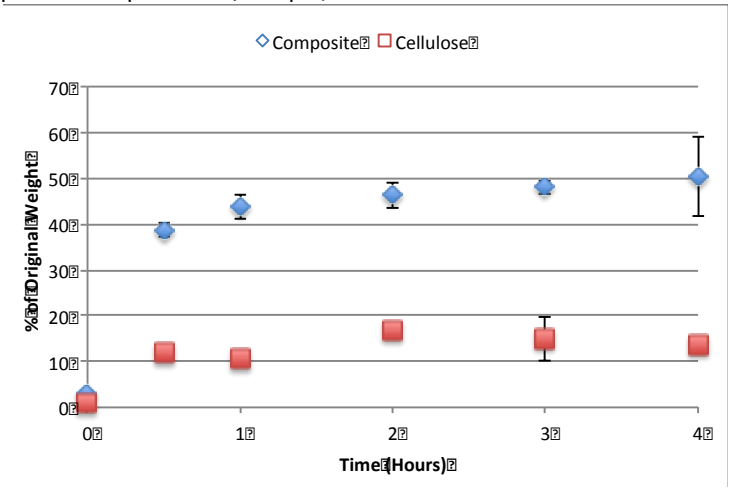
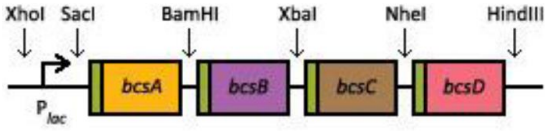


Figure 16: Cellulose Operon.



Finally, while the work presented to date involved a combination of recombinant *E. coli* strains carrying multiple plasmids and a cellulose matrix produced by *G. xylinus*, the ultimate goal is to combine all aspects of the system into a single organism. To that end, the cellulose genes were cloned into a single operon (**Figure 16**) to be expressed in a more genetically malleable organism than *G. xylinum*. Attempts to express the operon in *Agrobacterium tumefaciens* and *Pseudomonas putida* were not successful at the time of this report.

Summary:

Over the course of this award we have successfully completed the following objectives:

- Bacterial Cellulose Pellicles successfully utilized to entrap control detection organism
- Detection of GFP & RFP fluorescence demonstrated
- Tailorable fluorescence signal demonstrated through differential Loading of Detection Organism
- Genetic Amplifier Loop successfully implemented in control system
- Semi-quantitative determination of fluorescence system developed
- “Low-tech” visualization of cellulose detector achieved
- Stability of living membrane system for 30 days and more demonstrated
- Improved the rate and degree of rehydration after lyophilization
- Confirmed expression of AchE & AntR chimeric receptors
- Confirmed functional efficacy of AntR chimeric receptor in Tox system